REMARKS

Claims 26-50 are in this application. The Examiner objects to the numbering of the claims and points out that when new claims are presented, they must be numbered consecutively beginning with the number next following the highest numbered claims previously presented. Moreover, the Examiner assumes that since numbering of the claims in the Preliminary Amendment began with 26 that Applicants intended to cancel original claim 26. Applicants respectfully point out that original claims 1-26 were amended during the International stage. At that time, and in accord with the rules of practice governing the International stage, original claim 7 was deleted, and claims 8-26 were renumbered as claims 7-25, and all claims numbered 1-25 were provided on amended sheets bearing page numbers 26-29. Upon entry into the U. S. National stage, claims 1-25 were therefore pending. All of the these claims were canceled in the Preliminary Amendment dated March 20, 2002 and new claims 26-50, numbered consecutively beginning with the number next following the highest numbered claims previously presented, were added. Accordingly, Applicants' remarks will be based on claim numbering as set forth in the Preliminary Amendment and the Restriction Requirement mailed on June 20, 2003.

Applicants hereby cancel non-elected claims 36-40, 45 and 47-50. Accordingly, claims 26-35, 41-44 and 46, as set forth in the Preliminary Amendment dated 20 March 2002, are pending and under examination.

The Examiner asserts that a certified copy of the GB priority application has not been filed as required by 35 U.S.C. § 119(b), and therefore Applicants are not entitled to their claimed September 30, 1999 priority date. Applicants respectfully assert that a certified copy of the GB priority document was timely filed with the International Bureau of WIPO as acknowledged in the Notification Concerning Submission or Transmittal of Priority Document received from the International Bureau (a true copy of this Notification is attached hereto as exhibit A). Moreover, in the Notification of Missing Requirements Under 35 U.S.C. § 371 in the United States Designated/Elected Office mailed to Applicants on June 12, 2002 (a true copy of which is attached hereto as exhibit B), the U.S.P.T.O. acknowledged receipt of, *inter alia*, the priority document. Accordingly, Applicants timely took all steps required by the statutes, regulations and rules to be entitled to priority under 35 U.S.C. § 119(b). Applicants herewith provide a certified copy of the GB priority document for the Examiner's convenience (attached hereto as exhibit C).

Claim 31 stands rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement for recitation of an aluminum salt concentration of 0.4-1 micrograms per dose. Applicants have amended claim 31 consistent with the specification (see page 9, lines 19-20) and the claims as originally presented in the PCT application as filed. This obvious typographical error has now been corrected.

Claims 26-28, 32-34 and 41-43 are rejected under 35 U.S.C. § 103(a) as unpatentable over the combined teachings of Couch et al., Chaloupka et al and Kinster et al. Applicants traverse. As an intial matter, Applicants respectfully assert that, as described above, Applicants are entitled to the priorty date of the GB application (i.e., September 30, 1999). Accordingly, Kistner et al. is not available as a reference by virtue of its PCT publication date of March 23, 2000, approximately six months after Applicants' earliest priority.

The instant invention discloses and claims a monovalent influenza vaccine composition comprising an influenza virus component which is a low dose of egg-derived influenza virus antigen from an influenza virus strain that is associated with a pandemic outbreak, or has the potential to be associated with a pandemic outbreak, in combination with a suitable adjuvant, wherein the low antigen dose is less than 15 μg per combined dose of vaccine. The selection of a such a monovalent strain, associated with a pandemic outbreak, is nowhere suggested in the prior art.

Couch et al. merely suggests that influenza vaccines containing lower doses of antigen are desirable and could possibly be achieved through efforts such as adjuvant development, but provides no teaching of how to achieve such results. Couch et al. reports that while the current inactivated influenza virus vaccines are immunogenic and well tolerated, they appeared less effective for pandemic influenza in 1968 and in 1957, and that they need to be improved to provide better protection against pandemic and interpandemic influenza. Couch et al. suggest that this improvement may be achieved by i) increasing the doses of HA and NA (requiring an increase in supply), ii) using adjuvants or immunomodulators to lower the dose of antigen (see page S42, second paragraph), iii) using the mucosal (airborne) administration route (S41, right-hand column, first two paragraphs), or iv) by using purified subunit vaccines (S40, left-hand column, second full paragraph). Regarding option i), Couch et al. teach that there is a direct correlation between the dose and the mean titer that persisted for 24 weeks, and that a maximal response was not identified (S40, left-hand column, third full paragraph). Accordingly, Couch et al. teach the desirability and feasability in increasing dose of antigen to <u>increase</u> the magnitude and duration of the immune response, and therefore provides no motivation and in fact teaches away from efforts

to decrease dose. Regarding option ii) several different adjuvants have been used, including alum, oil-in-water emulsion, QS-21, MPL etc. (S40, right-hand column, third full paragraph) and are reported as not having a major enhancing effect in recent clinical human studies (S41, left-hand column, first paragraph). Accordingly, Couch et al. merely identify that what is needed is an improved influenza vaccine formulation which is effective against pandemic viruses, but provides no teaching of how to obtain such a formulation.

Chaloupka et al. is a review article comparing six then (1996) currently available European influenza vaccines, all containing three virus strains (usually two types A and one type B). Although there is said to be 15 μg HA/dose according to the vaccine manufacturers (page 126, right-hand column, second full paragraph), it is clear from Table 1 on page 123 and also on page 124, right-hand column, third full paragraph, that all influenza vaccines tested in fact contain a higher amount of HA, actually ranging from 33 μg/dose to 58 μg/dose. There is no discussion in Chaloupka et al. about pandemic influenza vaccines, as there are no suggestions of improved vaccine formulations which would be appropriate for pandemic situations.

The skilled artisan, faced with the teachings of Couch et al. and Chaloupka et al. would not reach the instant invention. That is, Chaloupka et al. does not solve the problem articulated by Couch et al. for the development of an influenza vaccine formulation which is effective against pandemic viruses, and certainly does not teach or suggest that this can be achieved using a vaccine formulation containing a low dose of antigen from a single strain (i.e., monovalent).

Indeed, it is known and ackowledged in Couch et al. that heterologous protection is less effective than homologous protection; i.e., the titers of serum anti-HA antibody and the degree of protection are lower when the infectious virus and the vaccine virus are antigenically distinct than when both are antigenically similar. Accordingly, the routine practice in this art has so far been to include several antigenic components (HA and NA typically) from more than one influenza virus strain, typically from three strains (see for example Chaloupka et al, page 121, right hand column, last sentence of first paragraph).

Furthermore, it is questionable that the disclosed compositions in the cited references are effective (i.e., protective) in human clinical studies. Indeed Couch et al. clearly report that although improved influenza compositions (with adjuvants) have been shown to enhance immune responses in animal models, they have failed to demonstrate a major enhancing effect in human studies (page S41, left-hand column, first paragraph). In contrast, Applicants were able to demonstrate that the improved vaccine formulation as claimed were not only

capable of inducing an immune response in humans but also that a protective titer was reached in all groups tested (see the instant specification, Example 4 on pages 21-24, and in particular lines 8-10 on page 24, and Example 5 on pages 24-25).

In summary, the cited prior art do not suggest the instant invention to one skilled in this art. Although Couch et al. identify the problem to be solved, and suggest several paths of research aimed at attempting to solve the problem, neither cited reference, alone or together, provides a solution that is reasonably expected to be successful. Accordingly, Applicants respectfully assert that the instant invention is not obvious in view of the cited prior art.

Claims 44 and 46 stand rejected as obvious in view of Couch et al. Chaloupka et al. and Kistner et al., and further in view of Riberdy et al. Applicants respectfully assert that since these claims depend on novel and inventive claims 26 and 41, respectively (see argument above), the obviousness rejection should be withdrawn.

Applicants respectfully request allowance of the instant application.

Respectfully submitted,

William R. Majarian
Attorney for Applicants
Registration No. 41,173

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EXHIBIT A

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

PRIVETT, Kathryn, Louise
Corporate Intellectual Property
SmithKline Beecham
Two New Horizons Court
Brentford
Middlesex TW8 9EP
ROYAUME-UNI

Date of mailing (day/month/year) 06 November 2000 (06.11.00)	
Applicant's or agent's file reference KLP/B45201	**IMPORTANT NOTIFICATION**
International application No. PCT/EP00/09509	International filing date (day/month/year) 27 September 2000 (27.09.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 30 September 1999 (30.09.99)
Applicant SMITHKLINE BEECHAM BIOLOGICALS S.A. et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
30 Sept 1999 (30.09.99)	9923176.3	GB	25 Octo 2000 (25.10.00)

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No. (41-22) 740.14.35	Authorized officer David Lopez-Ramirez Telephone No. (41-22) 338.83.38

EXHIBIT B

cc: KP

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents, Box PCT
United States Patent and Trademark Office
Washington, D.C. 20231
www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
10/088,632	Erik D'Hondt	B45201

INTERNATIONAL APPLICATION NO.	
PCT/EP00/09509	
I.A. FILING DATE	PRIORITY DATE
09/27/2000	09/30/1999

Glaxosmithkline
Corporate Intellectual Property - UW2220
PO Box 1539
King of Prussia, PA 19406-0939

CONFIRMATION NO. 7231

371 FORMALITIES LETTER

OC000000008256413

Date Mailed: 06/12/2002

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GLAXOSMITHKLINE
2002 JUN 21 A 10: 32

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as an Elected Office (37 CFR 1.495):

- U.S. Basic National Fees
- Priority Document
- Copy of IPE Report
- Copy of references cited in ISR
- Copy of the International Application
- Copy of the International Search Report
- Information Disclosure Statements
- Preliminary Amendments
- Request for Immediate Examination

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.
- **$130** Surcharge for providing the oath or declaration later than the appropriate 30 months months from the priority date (37 CFR 1.492(e)) is required.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTH FROM THE DATE OF THIS NOTICE OR BY 22 or 32 MONTHS (where 37 CFR 1.495 applies) FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

SUMMARY OF FEES DUE:

Total additional fees required for this application is **$130** for a Large Entity:

- **$130** Late oath or declaration Surcharge.

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

*A copy of this notice **MUST** be returned with the response.*

PAULETTE R KIDWELL

Telephone: (703) 305-3656

PART 1 - ATTORNEY/APPLICANT COPY

U.S. APPLICATION NUMBER NO.	INTERNATIONAL APPLICATION NO.	ATTY. DOCKET NO.
10/088,632	PCT/EP00/09509	B45201

FORM PCT/DO/EO/905 (371 Formalities Notice)

EXHIBIT C

The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed AnBrewer

Dated 2 October 2000

For Official use

THE PATENT OFFICE
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30 SEP 1999
LONDON

01OCT99 E480836-1 D02029
P01/7700 0.00 - 9923176.3

Your Reference: KP/RH/ B45201

9923176.3

The Patent Office

Request for grant of a Patent

Form 1/77 Patents Act 1977

Notes
Please type, or write in dark ink using CAPITAL letters. A prescribed fee is payable for a request for grant of a patent. For details, please contact the Patent Office (telephone 071-483 4700).

Rule 16 of the Patents Rules 1990 is the main rule governing the completion and filing of this form.

② Do not give trading styles, for example, 'Trading as XYZ company', nationality or former names, for example, 'formerly (known as) ABC Ltd' as these are not required.

Warning
After an application for a Patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977 and will inform the applicant if such prohibition or restriction is necessary. Applicants resident in the United Kingdom are also reminded that under Section 23, applications may not be filed abroad without written permission unless an application has been filed not less than 6 weeks previously in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction revoked.

① Title of invention

1 Please give the title of the invention: Novel Composition

② Applicant's details

❑ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate Name: SmithKline Beecham Biologicals S.A.

Country *(and State of incorporation, if appropriate)*: Belgium

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases, please given the following details:**

Address: Rue de l'Institut 89, B-1330 Rixensart

postcode *(if applicable)* -

Country: Belgium

ADP number *(if known)*: 5800974002

5781117001

2d, 2e and 2f: If there are further applicants please provide details on a separate sheet of paper

☐ **Second applicant *(if any)***

2d If you are applying as a corporate body please give:
Corporate Name

Country *(and State of Incorporation, if appropriate)*

2e If you are applying as an individual or one of a partnership please give in full:

Surname:

Forenames:

2f **In all cases,** please give the following details:

Address:

UK postcode *(if applicable)*
Country
ADP number *(if known)*

❸ *An address for service in the United Kingdom must be supplied*

Please mark correct box.

❸ Address for service details

3a Have you appointed an agent to deal with your application?

Yes ☒ No ☐ ➲ ***go to 3b***

please give details below

Agent's name	KATHRYN L PRIVETT
Agent's address	SmithKline Beecham Corporate Intellectual Property New Horizons Court Great West Road Brentford, Middlesex
Postcode	TW8 9EP
Agent's ADP number	7735830001

3b: If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

Address

Postcode

Daytime telephone number *(if available)*

ADP number *(if known)*

④ Reference number

4. Agent's or applicant's reference number *(if applicable)* KLP/RH/ B45201

⑤ Claiming an earlier application date

Please mark correct box

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

Yes ☐ No ☒ *go to 6*

please give details below

☐ number of earlier application or patent number

☐ filing date *(day month year)*

☐ and the Section of the Patents Act 1977 under which you are claiming:

Please mark correct box

15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

⑥ Declaration of priority

⑥ *If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number*

Please give the date in all number format, for example, 31/05/90 for 31 May 1990

6. If you are declaring priority from previous application(s), please give:

Country of Filing	Priority application number *(if known)*	Filing Date *(day, month, year)*

❼ Inventorship

Notes (left margin):
- *any applicant is not an inventor*
- *there is an inventor who is not*
- *an applicant, or*
- *any applicant is a corporate body.*

7. Are you (the applicant or applicants) the sole inventor or the joint inventors?

Please mark correct box

Yes ☐ No ☒ ➲ ***A Statement of Inventorship on Patents form 7/77 will need to be filed (see Rule 15).).***

❽ Checklist

❽ Please supply duplicates of claim(s), abstract, description and drawings).

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 —

Claim(s)	3	Description	19
Abstract		Drawing(s)	2 only

8b Which of the following documents also accompanies the application?

Please mark correct box(es)

Priority documents *(please state how many)* —

Translation(s) of Priority documents *(please state how many)* —

Patents Form 7/77 - Statement of Inventorship and Right to Grant —

Patents Form 9/77 - Preliminary Examination Report —

Patents Form 10/77 - Request for Substantive Examination —

❾ Request

❾ You or your appointed agent (see Rule 90 of the Patents Rules 1990) must sign this request.

I/We request the grant of a patent on the basis of this application.

Please sign here á

Signed Kathryn Privett Date: 30/09/99 *(day month year)*

KATHRYN L PRIVETT
Chartered Patent Attorney
Attorney for the Applicant

A completed fee sheet should preferably accompany the fee.

Please return the completed form, attachments and duplicates where requested, together with the prescribed fee to either;

☐ *The Comptroller or*	☑ *The Comptroller*
The Patent Office	*The Patent Office*
Cardiff Road	*25 Southampton Buildings*
Newport	*London*
Gwent	*WC2A 1AY*
NP9 1RH	

Novel Compositon

This invention relates to novel vaccine formulations, methods for preparing them and their use in prophylaxis or therapy. In particular the present invention relates to vaccines for administration during pandemics.

Influenza virus is one of the most ubiquitous viruses present in the world, affecting both humans and livestock, following a still unpredictable pattern of regular epidemics and irregular pandemics.

Although it is often considered to be a trivial disease, influenza can have a devastating impact. Outbreaks have been recorded throughout history. Over 30 worldwide epidemics or pandemics, are known to have occurred since 1580, four of them in this century.

The usual symptoms of influenza include cough, fever, headache and muscle pains. Many sufferers develop complications or secondary bacterial infections which can be very serious and even fatal.

During inter-pandemic periods, influenza viruses circulate that are related to those from the preceding epidemic. The viruses spread among people with varying levels of immunity from infections earlier in life. Such circulation, over a period of usually 2-3 years, promotes the selection of new strains which have changed enough to cause an epidemic again among the general population; this process is termed "antigenic drift". "Drift variants" may have different impacts in different communities, regions, countries or continents in any one year, although over several years their overall impact is often similar.

Typical influenza epidemics cause increases in incidence of pneumonia and lower respiratory disease as witnessed by increased rates of hospitalisation or mortality. The elderly or those with underlying chronic diseases are most likely to experience such complications, but young infants also may suffer severe disease.

At unpredictable intervals, novel influenza viruses emerge with a key surface antigen, the haemagglutinin, of a totally different sub-type from strains circulating the season before. This phenomenon is called "antigenic shift". It is thought that at least in the past pandemics have resulted when influenza from a different species, such as an avian or a porcine influenza, has transferred across the species barrier. If such viruses have the potential to spread readily from person to person, then more widespread and severe epidemics may occur, usually to a similar extent in every country within a few months to a year, resulting in a pandemic.

The features of an influenza virus strain that give it the potential to cause a pandemic outbreak are: it contains a new haemagglutinin compared to the heamagglutinin in the currently circulating strains; it is capable of being transmitted horizontally in the human population; and it is pathogenic in humans. A new haemagglutinin may be one which has not been evident in the population for a number of years, probably decades, such as H1. Or it may be a haemagglutinin that has not been circulating in the human population before, for example H5 or H9 which are both found in avian influenza. In either case the majority of the population will not previously have encountered the antigen and will not be immunologically well equipped to respond to it.

For the past 20 years the haemagglutinin in circulating human influenza viruses has been H2 or H3. Two years ago influenza with H5 (H5N1) which is an avian influenza virus appeared in people in Hong Kong. However the virus was not transmitted from person to person and so did not have the capability to result in a pandemic.

Certain parties are at an increased risk of becoming infected with flu in a pandemic situation . The elderly, the chronically ill and small children are particularly susceptible but many young and apparently healthy people are also at risk. It is important to be protected effectively as soon as possible and in a simple way.

Another group of people who are at increased risk are travellers. People travel more today than ever before and the regions where most new viruses emerge, China and South East Asia, have become popular travel destinations in recent years. This change in travel patterns enables new viruses to reach around the globe in a matter of weeks rather than months or years.

Thus for these groups of people there is a particular need for vaccination to protect against influenza in a pandemic situation or a potential pandemic situation.

A great deal of effort is being put into forming an effective international strategy for reacting to a pandemic situation and the World Health Organisation is instrumental in this. A key desirable measure is the development of a pandemic vaccine strategy and up to now this has not been achieved on the scale required to address a flu pandemic.

It has now been surprisingly found that vaccines that will be useful in a pandemic situation can be formulated quickly and in a specific manner. In particular it has been discovered that a low dose influenza virus vaccine containing purified virus adjuvanted with a traditional carrier, which can be produced quickly and economically enough to enable vaccination of populations on a large scale, is effective in humans.

In the past, egg-derived, poorly purified, whole inactivated influenza vaccine adjuvanted with aluminium salts has been used commercially. However, the product was rather reactogenic and the approach was abandoned at the end of the 1970s.

More recently, more highly purified, better characterised split influenza vaccines have been combined with adjuvants in an attempt to improve on the immunogenicity in adults and older people. In spite of significant increases in immune responses in mice, a number of approaches using new generation adjuvants could not be confirmed in man. In all of these studies, the regular 15 µg content of haemagglutinin antigen has been used to prepare the formulated vaccines.

A recent report (Kistner et al (1999) in *Inactivated Influenza Vaccines Prepared in Cell Culture, Dev Biol Stand. Basel, Karger.* Vol 98 pp 101-110) describes a primate study in which cell culture-derived non-purified vaccine containing three influenza strains mixed with $Al(OH)_3$ was given to chimpanzees. This induced a systemic response that was as good at a dose of 1.5 μg haemagglutinin per strain as at the standard 15 μg of haemmaglutinin per strain. This study was directed towards the goal of developing a Vero cell-derived influenza whole virus which fulfills all of the conventional requirements of the European Pharmacopoeia, the WHO and other regulatory organisations for an influenza virus vaccine.

For a standard influenza vaccine for routine use there may be difficulties associated with the use of aluminium salts as adjuvants. This is because influenza vaccines are intended for annual use and the repeated injections of Al^{3+} which would therefore be necessary may be undesirable. But for a pandemic situation that may occur only several times in a century, the use of Al^{3+} is not precluded.

The present invention therefore provides a vaccine composition comprising a low dose of influenza virus antigen from a single influenza virus strain that is associated with a pandemic outbreak or has the potential to be associated with a pandemic outbreak, in combination with a suitable adjuvant.

The vaccine of the present invention is provided at an effective dose to prevent influenza infection.

The vaccine formulations of the present invention will contain an immunoprotective quantity of the antigen and may be prepared by conventional techniques.

The vaccine compositions of the invention are preferably, but not necessarily, administered in a single dose.

The use of a low dose of antigen and the use of a single influenza strain (i.e. a monovalent vaccine) contribute to the speed required to react to a pandemic situation.

A low dose of influenza virus antigen in the composition according to the invention is a dose which is below the currently accepted dose for human influenza vaccines which is 10-15 µg of haemagglutinin antigen, normally 15 µg in accordance with regulations such as those issued by EMEA in Europe.

Thus, the low dose according to the invention is below 10 µg of haemagglutinin, preferably below 8 µg of haemagglutinin, more preferably between 0.1 and 7.5 µg of haemagglutinin, most preferably between 1 and 5 µg of haemagglutinin per vaccine dose. Preferably the dose is significantly lower than in conventional influenza vaccines to enable the production of significantly greater quantities of influenza vaccine for a pandemic situation than would be possible using current influenza vaccine at current dose levels. Equally the dose of antigen needs to be high enough to provide effective protection.

The influenza virus antigen in the vaccine composition according to the invention needs to be obtainable by a quick and efficient method to meet the needs of a pandemic vaccine. Currently the preferred method is by growing influenza virus in eggs and purifying the harvested allantoic fluid. Eggs can be accumulated in large numbers at short notice. Cell culture methods, such as growth of the virus on dog kidney cell lines such as MDCK or MDCK-like cells, or on Vero cells, may also be suitable. Cell culture methods are not currently preferred for a pandemic situation because of the need to maintain dedicated fermenters permanently ready to receive an inoculum of the relevant influenza strain.

The influenza virus in the vaccine composition is preferably in the form of whole virus particles, but may alternatively be split virus prepared by conventional methods.

An advantage of a whole virus vaccine over a split virus vaccine for a pandemic situation is that it avoids the uncertainty over whether a split virus vaccine can be successfully produced for a new strain of influenza virus. For some strains the conventional detergents used for producing the split virus can damage the virus and

render it unusable. Although there is always the possibility to use different detergents and/or to develop a different process for producing a split vaccine, this would take precious time, which may not be available in a pandemic situation.

In addition to the greater degree of certainty with a whole virus approach, there is also a greater vaccine production capacity than for split virus since there is a higher level of wastage of virus antigen in the process for preparing a split vaccine.

The use of an adjuvant in the vaccine composition according to the invention allows the use of a lower dose of virus antigen than in conventional vaccines.

Preferably the adjuvant in the composition according to the invention is an adjuvant which is readily available in large quantities. A particularly preferred adjuvant according to the invention contains at least one aluminium salt, most preferably a combination of aluminium hydroxide and aluminium phosphate. Preferably the aluminium hydroxide is present at a higher concentration per vaccine dose than the aluminium phosphate.

The total amount of aluminium salt per 0.5 or 1 ml dose of vaccine is normally in the range 0.4-1.0 mg.

In another aspect the invention provides a method for the production of an influenza vaccine for a pandemic situation which method comprises mixing an influenza virus antigen from a single influenza virus strain that is associated with a pandemic outbreak or has the potential to be associated with a pandemic outbreak, with a suitable adjuvant and providing vaccine lots which contain less than 10 μg influenza haemagglutinin antigen per dose.

In still another aspect the invention provides a process for purifying influenza virus antigen for use in a vaccine, which process comprises the step of treating a mixture containing the influenza virus antigen with a protease to digest non-influenza virus proteins.

The purification is carried out on a preparation of influenza virus harvested from a culture. Surprisingly, the influenza virus particles are resistant to the protease digestion step. A preferred protease for use in the method is trypsin which is preferably used at a concentration of between 0.1 – 10 µg/ml pure trypsin. Alternative protease enzymes that may be used in include plasmin and chymotrypsin.

Normally, the protease digestion step is performed after the influenza virus antigen has been partially purified by one or more physical separation steps such as centrifugation and filtration. Where the desired product is a whole virus vaccine, the protease digestion step is carried out prior to a virus inactivation step.

The purification method according to the invention can be successfully used to provide purified influenza virus antigen in the form of split or whole virus substantially free of contaminating host cell proteins, suitable for use in a vaccine.

The term "substantially free of contaminating host cell proteins" means that less than 10%, preferably less than 8% and more preferably less than 5% of the total protein is host cell protein as detected by scanning of Coomassie-stained polyacrylamide gels. In the case of influenza cultured in eggs, the predominant host protein is ovalbumin which makes up about 60-70% of the total protein mass of the allantoic fluid. Preferably ovalbumin is present in the purified influenza virus preparation at a concentration of less than 1%, more preferably less than 0.1% and most preferably only about 0.05% of the total protein content as assessed by scanning stained gels.

In a further aspect the invention provides the use of a dose of below10 µg, or below 8 µg, or from 1 - 7.5 µg, or from 1 – 5 µg of influenza virus haemagglutinin antigen from a single strain of influenza associated with a pandemic outbreak or having the potential to be associated with a pandemic outbreak, in the manufacture of a vaccine for the prevention of influenza.

Alternative adjuvants which are suitable for use in the vaccine composition according to the invention include a range of adjuvants capable of enhancing the immune response to virus antigens.

3 De-O-acylated monophosphoryl lipid A (3D-MPL) is one such adjuvant. This is described for example in GB 2220211 (Ribi). Chemically it is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in EP 0 689 454. The preferred form of 3D-MPL is particles of no greater than 120 nm, normally 60-120 nm, preferably about or less than 100 nm in diameter (as described in EP 0 689 454).

3D-MPL will usually be present in the range of 10 μg – 100 μg, preferably 25-50 μg per dose wherein the antigen will typically be present in a range 2-50 μg per dose.

Another suitable adjuvant is QS21, which is an HPLC-purified, non-toxic fraction of a saponin from the bark of the South American tree Quillaja Saponaria Molina. Optionally this may be admixed with 3D-MPL, optionally together with an carrier.

A method for producing QS21 is described in US 5,057,540.

Non-reactogenic adjuvant formulations containing QS21 are also suitable for use in the vaccine compositions according to the invention and are described for example in WO 96/33739. Such formulations comprising QS21 and cholesterol have been shown to be successful adjuvants when formulated together with an antigen.

Combinations of different adjuvants, such as those mentioned hereinabove, are also contemplated as providing an adjuvant which is suitable for use in the invention. For example, QS21 can be formulated together with 3D-MPL. The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1 : 5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5 : 1 to 1 : 1 3D-MPL: QS21.

Advantageously the vaccine compositions according to the invention may be formulated with a carrier, usually in combination with one of the alternative adjuvants described above The carrier may be for example an oil in water emulsion, or an aluminium salt.

A preferred oil-in-water emulsion comprises a metabolisible oil, such as squalene, alpha tocopherol and Tween 80. Additionally the oil in water emulsion may contain span 85 and/or lecithin.

In a preferred aspect aluminium hydroxide or aluminium phosphate will be added to the composition of the invention to enhance immunogenicity.

Typically for human administration QS21 and 3D-MPL will be present in a vaccine in the range of 1 μg - 200 μg, such as 10-100 μg, preferably 10 μg - 50 μg per dose. Typically the oil in water emulsion will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% Tween 80. Preferably the ratio of squalene to alpha tocopherol is equal to or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

Non-toxic oil in water emulsions preferably contain a non-toxic oil, e.g. squalane or squalene, an emulsifier, e.g. Tween 80, in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

A particularly potent alternative adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210.

The invention will now be further described in the following examples.

EXAMPLES

Example 1 – Preparation of monovalent bulk for split influenza vaccine

The vaccine bulk was prepared according to the flow sheet shown in Figure 1A. Figure 1B shows a generalised flow sheet for the purification process, including the optional trypsin incubation step.

Production of crude monovalent whole virus

Preparation of virus inoculum

On the day of inoculation of embryonated eggs a fresh inoculum is prepared by mixing the working seed lot with a phosphate buffer containing gentamycin sulphate at 0.5 mg/ml and hydrocortison at 25 μg/ml. (virus strain-dependent)
The virus inoculum is kept at 2-8°C.

Inoculation of embryonated eggs

Nine to eleven day old embryonated eggs are used for virus replication.
The eggs are incubated at the farms before arrival at the manufacturing plant and transferred into the production rooms after decontamination of the shells.
The eggs are inoculated with 0.2 ml of the virus inoculum on an automatic egg inoculation apparatus.

The inoculated eggs are incubated at the appropriate temperature (virus strain-dependent) for 48 to 96 hours. At the end of the incubation period, the embryos are killed by cooling the eggs and stored for 12-60 hours at 2-8°C.

Harvest

The allantoic fluid from the chilled embryonated eggs is harvested by appropriate egg harvesting machines. Usually, 8 to 10 ml of crude allantoic fluid can be collected per egg. To the crude monovalent virus bulk 0.100 mg/ml thiomersal is added (in an alternative method, thiomersal is not added).

Concentration and purification of whole virus from allantoic fluid

1. Clarification

The harvested allantoic fluid is clarified by moderate speed centrifugation (range: 4000 – 14000 g).

2. Adsorption step

To obtain a $CaHPO_4$ gel in the clarified virus pool, 0.5 mol/L Na_2HPO_4 and 0.5mol/L $CaCl_2$ solutions are added to reach a final concentration of $CaHPO_4$ of 1.5 g to 3.5 g $CaHPO_4$/litre depending on the virus strain.

After sedimentation for at last 8 hours, the supernatant is removed and the sediment containing the influenza virus is resolubilised by addition of a 0.26 mol/L EDTA-Na_2 solution, dependent on the amount of $CaHPO_4$ used.

3. Filtration

The resuspended sediment is filtered on a 6µm filter membrane.

4. Sucrose gradient centrifugation

The influenza virus is concentrated by isopycnic centrifugation in a linear sucrose gradient (0.55 %). The flow rate is 8 – 15 litres/hour.

At the end of the centrifugation, the content of the rotor is recovered by four different fractions (the sucrose is measured in a refactometer):

- fraction 1 55-52% sucrose
- fraction 2 approximately 52-38% sucrose
- fraction 3 38-20% sucrose*
- fraction 4 20- 0% sucrose

* virus strain-dependent: fraction 3 can be expended to 15% sucrose.

For further vaccine preparation, only fractions 2 and 3 are used.

Fraction 3 is washed by diafiltration in order to reduce the sucrose content to approximately 6%. The influenza virus present in this diluted fraction is pelleted to remove soluble contaminants.

The pellet is resuspended and thoroughly mixed to obtain a homogeneous suspension. Fraction 2 and the resuspended pellet of fraction 3 are pooled and phosphate buffer is added to obtain a volume of 40 litres.

At this stage, the product is called "monovalent whole virus concentrate".

Sterile filtration

The whole virus material is filtered on filter membranes ending with a 0.2 μm membrane. At the end of the filtration, the filters are washed with phosphate buffer containing 0.025 % TWEEN 80. As a result, the final volume of the filtered fraction 2 is 5 times the original fraction volume.

Inactivation

The filtered monovalent material is incubated at 22 ± 2°C for at most 84 hours (dependent on the virus strains, this incubation can be shortened). After this incubation time, phosphate buffer containing 0.025% TWEEN 80 is added in order to reduce the total protein content down to max. 250 μg/ml. Formaldehyde is added to a final concentration of 250 μg/ml and the inactivation takes place at 20°C ± 2°C for at least 72 hours.

Final sterile filtration

The inactivated material is diluted to approximately 500 μg protein and 0.05% TWEEN, prefiltered on membranes ending with 0.8 μm and finally filtered on membranes ending with 0.2μm.

Depending on the virus strain the last filtration membrane can be 0.8 μm. At this stage, the product is called: "monovalent final bulk".

Storage

The monovalent final bulk is stored at 2 – 8°C for a maximum of 18 months.

Purity

Purity was determined by O.D. scanning of Coomassie-stained polyacrylamide gels. Peaks were determined manually. Results are given in the table below:

Viral Proteins (HA, NP, M) %					Other viral and host-cell derived proteins %
H3N2	**HA dimer**	**HA1 + 2**	**NP**	**M**	
A/Syd/5/97 A/Nan933/95	1034 8.17	22.34 15.8	25.16 40.09	37.33 30.62	4.83 5.32
B					
B/Har/7/94 B/Yam/166/98	5.71[2] 0.68	24.07 27.62	15.64 21.48	50 46.02	4.58 4.2
H1N1					
A/Tex/36/91 A/Bei/262/95		33.42 32.73	24.46 35.72	34.33 27.06	7.79 4.49
H2N2					
A/sing/1/57	2.8	39.7	21.78	32.12	3.6

[1] = 100 % minus all non-identified peaks

Alternative method involving trypsin step

Trypsin digestion

After the sterile filtration step, the sterile material is subjected to a trypsinisation step. Pure trypsin for example commercially available pure porcine trypsin having a specific activity of 10,000 to 15,000 units/mg is added at a final concentration of 0.1-10 μg/ml. The mixture is incubated for 2 hrs at 37°C, stirring gently. The material is then refrigerated to cool for further processing.

Ultrafiltration

After trypsin digestion, the material may be subjected to ultrafiltration either before or after inactivation (as described above).

The virus material is ultrafiltrated on membranes with a mean exclusion limit of 20,000 to 50,000 D. During ultrafiltration, the content of formaldehyde, Nadoc and sucrose is considerably reduced.

After a first 4 fold volume reduction the volume remains constant during ultrafiltration (diafiltration) by adding phosphate buffer and phosphate buffered saline containing 0.01% TWEEN 80.

Results

Influenza whole virus vaccine prepared according to the trypsin method was observed on Coomassie-stained polyacrylamide gels. The viral proteins migrated to the same position as viral proteins which had not undergone a trypsin digestion step, indicating that the viral proteins had not been protease digested.

Example 2 – Preparation of vaccine doses from bulk vaccine

Final vaccine is prepared by mixing final bulk vaccine prepared as described in Example, with adjuvant mix and final buffer in such a way that the targeted antigen content is obtained and a concentration of 0.5 mg of Al salts is achieved per dose. The buffer used contains several salts, as listed below. The adjuvant is a mix of $AlPO_4$ and $Al(OH)_3$ and is used in a proportion of 3.6 mg of $AlPO_4$ and 0.4 mg of $Al(OH)_3$ per 4 mg/ml of stock solution.

Buffer composition:

Distilled water	0,800 l
NaCl	7,699 g
KCl	0,200 g
$MgCl_2.6H_2O$	0,100 g
$Na_2HPO_4.12H_2O$	2,600 g
KH_2PO_4	0,373 g

made up to a final volume of 1 litre with distilled water.

The procedure is as follows:

1. Use adjuvant mix at 10-15°C.
2. Add final vaccine buffer at 15-20°C and gently mix with magnetic stirrer.
3. While mixing add the appropriate bulk vaccine at 5-10°C.
4. Continue mixing for 10 to 30 minutes at room temperature.
5. Move adsorbed vaccine to cold room waiting for filling.
6. Final vaccine volume is 0.5 ml per dose.

Example 3 –Clinical data - low dose split influenza vaccine adjuvanted with aluminium salts

The following data come from a clinical trial in which a trivalent flu vaccine was prepared according to the general manufacturing outline for the commercially available Fluarix (Trade Mark) vaccine (which is a split flu vaccine). In practice, final trivalent bulk material was mixed with aluminium adjuvant as described in Example 2. Several different HA dosages were prepared.

The vaccine lots were tested in two age populations, 18-60 years and > 60 years, at 1.8 µg per dose per strain and 3.75 µg per dose per strain. 50 volunteers were vaccinated in each group.

The data corresponding to doses of 1.8 and 3.75 µg per strain are presented in the tables below.

	Adsorbed vaccine 3.75 µg/dose/strain			Adsorbed vaccine 1.8 µg/dose/strain		
	H_1N_1	H_3N_2	B	H_1N_1	H_3N_2	B
Seroconversion factor						
< 60 y	5	4.2	2.8	3.5	3.6	2.0
> 60 y	3.1	3.2	1.6	2.5	3.0	1.8
Seroconversion rate %						
< 60 y	57	5.5	28	51	45	24
> 60 y	44	4.4	13	38	38	13
Protection rate %						
< 60 y	89	87	100	82	76	98
> 60 y	81	71	100	64	67	100

Protective rates (%) in 18 – 60 year age groups				
	3.75 µg/dose/strain		1.8 µg/dose/strain	
	Pre	Post	Pre	Post
Against H_1N_1	43	89	45	82
Against H_3N_2	40	87	24	76
Against B	85	100	82	98

EU criteria are as follows:

- Seroconversion factor > 2,5
- Seroconversion rate > 40%
- Protection rate after vaccination > 70%

From the data in the tables it can be concluded that the EU criteria for seroconversion factor, seroconversion rate and protection rate are exceeded in the 2 age populations for the 2 different dosages tested against the A strains of influenza.

The protection rates against the B virus were over 80 and 90% before vaccination in the 2 study groups respectively. This pre-vaccination seropositivity to the B strain affects the vaccine response negatively. In spite of this, the antibodies to the B strain doubled after vaccination resulting a close to 100% protection rate.

Thus, a vaccine formulated with less than 4 µg of HA per strain and aluminium adjuvant has an acceptable reactogenicity profile and can induce an immune response that is in full compliance with all three EU criterial in the two study populations. Based on the observations made in this trial, it can be concluded that the low dose adsorbed vaccine is suitable for use in a pandemic situation.

Example 4 –Reactogenicity profile of a of low dose monovalent whole virus vaccine, purified and adsorbed on aluminium salt

Whole influenza vaccine was prepared according to Example 1 and Figure 1 (non-trypsin method).

At the purification stage for purifying the whole virus, besides the generally applied sucrose gradient centrifugation, the selected virus rich fraction was pelleted to remove more efficiently egg-derived contaminants.

Whole virus was inactivated with formaldehyde at a concentration of 250 µg/ml (compared to the inactivation process for split vaccine which is achieved by a combination of NADOC and exposure to formaldehyde at 50 µg/ml).

Once purified and inactivated, the antigen was adsorbed to a mix of aluminium hydroxide and phosphate at a concentration of 0.45 mg and 0.05 mg per dose respectively.

The purity was far superior to the purity of the whole virus adjuvanted vaccines of the past, in which plain allantoic or diluted allantoic fluid was used.

The antigen content of the whole virus was 7.5µg/dose of A/Sydney/5/97. This dosage was selected as a worst case scenario (as the highest antigen dosage that might be selected for a pandemic monovalent vaccine) to investigate for the upper limit of reactogenicity.

Based on the observations in Example 3 and the fact that whole virus is at least as immunogenic as split vaccine, it is most probably the case that a lower antigen dose will be used.

The reactogenicity, mainly the local events observed after vaccination were used to make a statistical comparison with historical data on Fluarix, the SmithKline Beecham Biologicals split Influenza vaccine.

The local reactions were selected for the comparison because they can be accurately measured and they are most indicative for a local reaction following an aluminium adjuvant containing vaccine.

Scope	Monovalent non adsorbed splitvaccine A/Sydney (15 µg)	Monovalent non adsorbed splitvaccine A/Sydney (7.5 µg)	Monovalent adsorbed splitvaccine A/Sydney (7.5 µg)	Monovalent adsorbed whole vaccine A/Sydney (7.5 µg)
(planned 4 x 50 n=200) n=196	n=48	n=49	n=50	n=48

RESULTS
(%)

Local and systemic reactions	23%	2%	32%	42%
Systemic reactions	17%	6%	6%	6%
Local reactions	27%	33%	42%	19%
Without reactions	33%	39%	20%	33%

The Mann-Whitney U test is a statistical test for comparing 2 populations and to test the zero hypothesis that two populations of results have identical distribution functions against the alternative hypothesis that the two distribution functions differ only with respect to location (median), if at all.

The outcome of the comparison of the reactogenicity of the monovalent low dose whole virus adjuvanted vaccine to results of clinical trials on Fluarix (Trade Mark) in 1996, '97 and '99 shows that there is no significant difference at the P 0.05 level.

This observation supports the use of whole virus adjuvanted vaccine, even at an antigen dosage higher than the dosage that is sufficient to induce high protection rates against Influenza.

CLAIMS

1. A vaccine composition comprising an influenza virus component which is a low dose of influenza virus antigen from a single influenza virus strain that is associated with a pandemic outbreak or has the potential to be associated with a pandemic outbreak, in combination with a suitable adjuvant.

2. A vaccine composition according to claim 1 wherein the influenza virus component is in the form of whole influenza virus.

3. A vaccine composition according to claim 1 or claim 2 wherein the adjuvant is an aluminium salt or salts.

4. A vaccine composition according to claim 3 wherein the adjuvant is aluminium hydroxide and aluminium phosphate.

5. A vaccine composition according to any one of claims 1 to 4 wherein the low antigen dose is less than 10 μg of haemagglutinin per dose of vaccine.

6. A vaccine composition according to claim 5 in which the low antigen dose is less than 8 μg of haemagglutinin per dose of vaccine.

7. A vaccine composition according to claim 6 in which the antigen dose is between 0.1 and 7.5 μg, or between 1 and 5 μg of haemagglutinin per dose of vaccine.

8. A vaccine composition according to any one of claims 1 to 7 wherein the influenza virus component is substantially free of host cell contamination.

9. A vaccine composition according to any one of claims 1 to 8 wherein the influenza virus component is purified by a method which includes a protease incubation step to digest non-influenza virus proteins.

10. A method for the production of an influenza vaccine for a pandemic situation which method comprises mixing a low dose of influenza virus antigen from a single influenza virus strain that is associated with a pandemic outbreak or has the potential to be associated with a pandemic outbreak, with a suitable adjuvant and providing vaccine lots which contain less than 10 μg influenza haemagglutinin antigen per dose.

11. A method according to claim 10 wherein the method includes a purification process for purifying the antigen.

12. A method according to claim 11 or claim 12 wherein the influenza virus antigen is in the form of whole influenza virus particles.

13. A process for producing influenza virus antigen for use in a vaccine, which process comprises the step of incubating a mixture containing influenza virus particles with a protease to digest non-influenza virus proteins.

14. A method according to claim 13 wherein the protease digestion step is performed after the influenza virus antigen has been partially purified by one or more physical separation steps.

15. A method according to claim 13 or claim 14 wherein the protease digestion step is performed prior to a virus inactivation step.

16. A method according to claim 15 wherein the purification process comprises the steps of:

(i) providing a harvested mixture of cultured influenza virus and host proteins from a culture;

(ii) partially purifying the influenza virus in the mixture by one or more physical purification steps;

(iii) performing a protease digestion step on the partially purified mixture to digest host proteins;

(iv) inactivating the influenza virus;

(iv) further purifying the influenza virus by at least one filtration step.

17. The use of a dose of below10 µg, or below 8 µg, or from 1 - 7.5 µg, or from 1 – 5 µg of influenza virus haemagglutinin antigen from a single strain of influenza associated with a pandemic outbreak or having the potential to be associated with a pandemic outbreak, in the manufacture of a vaccine for the prevention of influenza.

Fig 1 A

Preparation of inoculum

⇩

Inoculation of embryonated eggs

⇩

Incubation

⇩

Harvest

⇩

Crude monovalent whole virus bulk

⇩

Clarification

⇩

Adsorption

⇩

Filtration

⇩

Flow through centrifugation

⇩

Fraction section

⇩

Monovalent purified whole virus pool

⇩

Sterile filtration

⇩

Inactivation

⇩

Final filtraion

⇩

Monovalent final bulk

⇩

Storage

Fig 1 B

Down stream process

↓

Infected allantoic fluid

↓

Clarification by centrifugation
(500 - 1500 g)

↓

Adsorption on $CaHPO_4$ - gel

↓

Elution by EDTA

↓

Centrifugation over linear sucrose gradient

↓

Collect fractions

↓

Sterile filtration

↓	↓
Trypsin digestion 2 hrs / 37°C	Inactivation 20° ± 2°C, 72 hrs, 250 µg/ml formaldehyde
↓	↓
Ultrafiltration Kd = 30 - 50 000	
↓	↓
Inactivation 20° ± 2°C, 72 hrs, 250 µg/ml formaldehyde	
↓	↓
Sterile filtration	Store at 2 - 8°C